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A dileucine motif targets MCAM-l cell adhesion molecule to the basolateral membrane in MDCK cells

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Abstract Melanoma cell adhesion molecule (MCAM), an adhesion molecule belonging to the Ig superfamily, is an endothelial marker and is expressed in different epithelia. MCAM is expressed as two isoforms differing by their cytoplasmic domain: MCAM-l and MCAM-s (long and short). In order to identify the respective role of each MCAM isoform, we analyzed MCAM isoform targeting in polarized epithelial Madin–Darby canine kidney (MDCK) cells using MCAM-GFP chimeras. Confocal microscopy revealed that MCAM-s and MCAM-l were addressed to the apical and basolateral membranes, respectively. Transfection of MCAM-l mutants established that a single dileucine motif (41-42) of the cytoplasmic domain was required for MCAM-l basolateral targeting in MDCK cells. Although double labelling experiments showed that MCAM-l is not a component of adherens junctions and focal adhesions, its expression on basolateral membranes suggests that MCAM-l is involved in epithelium insuring.

Keywords: Focal adhesion; Targeting; Cadherins; Adhesion; MCAM

1. Introduction

Melanoma cell adhesion molecule (MCAM)/CD146 is a 113 kDa cell adhesion glycoprotein belonging to the Ig superfamily [1–3]. Its extracellular domain consists in 5 Ig domains (V–V–C2–C2–C2), a transmembrane domain and a cytoplasmic region [1]. MCAM presents homophilic interactions but also interacts with heterophilic ligands [4–8].

Human MCAM/CD146 was first identified as a melanoma progression antigen [9,10]. MCAM is also a differentiation marker of intermediary placental trophoblast, and is expressed in mammary lobular and ductal epithelium [11–14]. Endothelia and smooth muscle cells of blood vessels express also strongly MCAM [3,6,15]. CD146, detected in endothelial progenitors such as angioblasts or mesenchymal stem cells, is used as a marker of the endothelial lineage [16] and is involved in angiogenesis and vascular development [3,17,18]. MCAM presented a complex expression pattern in endothelial cells being located at cell–cell junction but also on apical membranes [15].

However these studies did not take into account that MCAM is expressed as two isoforms differing by the cytoplasmic region generated by alternative splicing of exon 15 [2,6,19]. These isoforms are named MCAM-l and MCAM-s for long and short cytoplasmic tail which are 21 and 43 aminoacid long, respectively. They share 16 aminoacids including a putative PKC phosphorylation site. Due to a splice-induced frameshift, MCAM-s exhibits a specific C-terminus that might interact with a PDZ domain. MCAM-l specific cytoplasmic domain contains an additional PKC site, a dileucine motif, and a YXXL motif which are conserved in vertebrates [2]. Most MCAM positive cells express both isoforms and generally MCAM-l is more prominent than the MCAM-s isoform [2,6], (Guezguez et al., submitted for publication). In this report, we show that in polarized epithelial Madin–Darby canine kidney (MDCK) cells, the MCAM-s and MCAM-l isoforms are addressed to apical and basolateral membranes, respectively. MCAM-l basolateral targeting requires a cytoplasmic dileucine motif and double labelling experiments showed that MCAM-l is not located in adherens junctions and focal adhesions.

2. Materials and methods

2.1. cDNA and plasmid constructs

To obtain the MCAM-GFP construct, the chicken MCAM-l (long isoform) and MCAM-s (short isoform) were amplified by polymerase chain reaction (PCR) using specific primers as described previously [6]. Then, the cDNAs were inserted in a pcDNA3-GFP vector (Clontech, Palo Alto, CA), placing the MCAM sequence in frame with sequence encoding GFP at the C-terminus.

Recombinant sequences encoding mutated cytoplasmic and extracellular domain of MCAM-l were obtained by PCR using specific primers (see Table 1). The PCR products were subcloned in the pCR®II-Topo® (Invitrogen Inc., UK). Recombinant sequences of MCAM-l mutated for extracellular regions were inserted in the pCR®II-Topo® placing the mutated MCAM sequence in frame with peptide signal sequence. Then, the cDNAs were digested with *Hin*dIII/*Bam*HI and inserted in a pcDNA3-GFP vector as described above.

The MCAM-l mutant in which the two Leucine (residues 603 and 604) were substituted by arginine and methionine residues-hereafter termed MCAM (LL/RM), was constructed by in vitro mutagenesis using Quick-change XL® site directed mutagenesis kit according to supplier's instructions (Stratagene, La Jolla, CA, USA). Briefly, complementary primers (22 nM) and 50 ng of MCAM-l-GFP cDNA

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Abbreviations: MCAM, melanoma cell adhesion molecule; C2, C2C12 mouse myogenic cell line; MDCK, Madin–Darby canine kidney cell line

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Table 1
Primers used for mutagenesis and RT-PCR experiments

cDNA	Expt	Amplicon size (bp)	Primer sequences
MCAM-long	PCR	1878	(F) 5′-CCCAAGCTTGGCAGCGAGCATGGCTGG-3′ (R) 5′-CGGGATCCCGGTTTCTCAGATCGATGTATTTC-3′
MCAM-short	PCR	1752	(F) 5′-CCCAAGCTTGGCAGCGAGCATGGCTGG-3′ (R) 5′-CGGGATCCCGGATCGATGTATTTCTCGCTATG-3′
MCAM-lΔ59	PCR	1860	(F) 5′-CCCAAGCTTGGCAGCGAGCATGGCTGG-3′ (R) 5′-ATAGGATCCCGCTCGCTCTGGTCAGC-3′
MCAM-lΔ45	PCR	1806	(F) 5′-CCCAAGCTTGGCAGCGAGCATGGCTGG-3′ (R) 5′-ATAGGATCCAAACCCTGCAGGAGCCCCGC-3′
MCAM-lΔ41	PCR	1806	(F) 5′-CCCAAGCTTGGCAGCGAGCATGGCTGG-3′ (R) 5′-ATAGGATCCAACCCCGCCTCTTCGGAAAG-3′
MCAM-lΔ30	PCR	1773	(F) 5′-CCCAAGCTTGGCAGCGAGCATGGCTGG-3′ (R) 5′-ATAGGATCCAATTCAACTACATTCTTGTC-3′
MCAM-Δcyto	PCR	1686	(F) 5′-CCCAAGCTTGGCAGCGAGCATGGCTGG-3′ (R) 5′-CGGGATCCCGCAGGAAGTAAATGATGGAGCC-3′
MCAM (LL/RM)	PCR	1878	(F) 5′-TCCGAAGAGGCGGGG**CGAATG**CAGGGTGCCAACGGC-3′ (R) 5′-GCCGTTGGCACCCTG**CATTCG**CCCCGCCTCTTCGGA-3′
MCAM-lΔext(12)	PCR	1250	(F) 5′-ATGTCTACGTGAACGTGAACGTCACTGTGTTC-3′ (R) 5′-ATTTAGGTGACACTATA-3′
MCAM-lΔext(345)		1080	(F) 5′-CTAGCTAGCATCATCATCGTGGCCATCATC-3′ (R) 5′-ATTTAGGTGACACTATA-3′
MCAM-lΔext(12345)	PCR	450	(F) 5′-ATGTCTACCCATCGGAGAGCAAAGGC-3′ (R) 5′-ATGGATCCGAACACAGTGAC-3′
MCAM-Δext(345)Δcyto	PCR	820	(F) 5′-CTAGCTAGCATCATCATCGTGGCCATCATC-3′ (R) 5′-CGGGATCCCGCAGGAAGTAAATGATGGAGCC-3′

Primer design was based on previous studies [2,6]. Mutated nucleotides leading to LL mutation into RM are bold and underlined.
(F): forward, (R): reverse.

template were used in PCR under the following conditions: 18 cycles of denaturation for 50 s at 95 °C and primer annealing and extension at 68 °C for 15 min. the primers used (mutated nucleotides are underlined) were shown in Table 1.

2.2. Cells, culture conditions and MDCK polarization

The mouse myogenic C2 cell line [20] and MDCK cells were grown in Dulbecco's modified Eagle medium (DMEM) plus Glutamax® supplemented with 10% heat-inactivated fetal calf serum and antibiotics (Gibco Life Technologies Inc. UK). All cultures were performed in 5% CO_2 humidified atmosphere at 37 °C. Before culture, glass coverslips 22 × 22 mm (CML, France) were placed in 6-well plate (TPP, Switzerland), sterilized by pure ethanol and air-dried at laminar flow of tissue culture cabinet for 2 h. For terminal polarization, MDCK cells were seeded at confluence in glass coverslips in culture medium and cultured for 5–8 day. The cell polarization was controlled by refringency of monolayer junctions with inverted phase-contrast microscope.

2.3. Cell transfection and electroporation

Transfected L929 and MDCK cells expressing wild-type and mutated MCAM-GFP were obtained after transfection of the relevant expressions vectors using Lipofectamine® 2000 reagent according to the manufacturer's recommendation (Invitrogen Inc.). Transfected cells were maintained in culture medium supplemented with 1 mg/ml neomycin (G418). Positive cells were detected and some of them were confirmed with sorted GFP populations using Phycoerythrine conjugated Anti-chicken MCAM (clone 264) by flow cytometry (Coulter Epics flow cytometer; Beckman Coulter, Fullerton, CA, USA) and by Western blotting of postnuclear lysates. Cells were then used either for immunofluorescence or confocal imaging.

For transient expression, 5×10^6 C2 cells were electroporated (Easyject Plus, Equibio, Ashford, UK) with 35 μg of MCAM-GFP or N-Cad-GFP expression vectors under 260 V, 1500 μF in 400 μL DMEM plus 15 mM HEPES, pH 7.2 (Invitrogen Inc.). The transfection efficiency was around 70% in all cases and the transfected cells were easily identified by GFP expression with fluorescence microscopy.

2.4. Antibodies and immunocytochemistry

Phycoerythrine conjugated monoclonal Anti-chicken MCAM (clone 264, [6]) was purchased from Southern Biotechnology and used for flow cytometry analyses.

For immunocytochemistry, MDCK transfected GFP cells were fixed with prewarmed 4% PBS-formaldehyde for 15 min. Thereafter, cells were treated with 0.1% PBS-Triton for 10 min and soaked in blocking solution (PBS containing 5% BSA) for 30 min. Coverslips were incubated with monoclonal anti-E-cadherin (1/400 dilution, clone 36, BD Biosciences) or monoclonal anti-ZO-1 (1/250 dilution, clone 1A12, Zymed Laboratories) in 1% PBS-BSA for 1 h. Then, washed and incubated with goat TRITC or Alexa Fluor® 555-conjugated anti-mouse antibodies (1/300 dilution, Molecular Probes).

C2 transfected GFP cells were incubated either with polyclonal anti-β-catenin (1/500 dilution, Sigma) or polyclonal anti-phospho-FAK (1/200, Santa Cruz Biotechnology) and then revealed with goat Alexa Fluor® 546-conjugated anti-polyclonal antibody (1/800 dilution, Molecular Probes). For positive controls, the focal contacts were revealed by anti-Rat β1 integrin (1/100, clone 9EG7, BD Pharmigen).

For cytoskeleton staining of L929 transfected GFP cells, TRITC-conjugated phalloidin (1/1500 dilution, Sigma) were used to visualize F-actin.

All procedures were performed at room temperature. Samples were mounted in Immuno-mount® (Thermo Shandon, Pittsburgh, USA) and analysed with TCS-SP confocal microscope (Leica, Mannheim,

Germany) set on sequential mode. The images were treated with the Metamorph software (Roper Scientific, Trenton, NJ) and Adobe Photoshop software (Adobe Systems, USA).

2.5. C2 cell adhesion assay

Glass coverslips were treated with 20% nitric acid, washed in methanol–acetone and coated with organopolysiloxane (Sigmacote, Sigma, Germany). Air-dried salinized coverslips were loaded with anti-mouse Fcγ fragment antibodies (Jackson ImmunoReasearch, West Grove, PA, USA) at 1 μg/cm^2 in 0.1 M borate buffer pH 8.0 for at least 5 h. Coverslips were then incubated for 2 h with a concentration of 5–10 μg/cm^2 (as determined by dot blot analysis) of purified Ncad-Fc chimera (extra-cellular domain of the chicken N-cadherin fused to the mouse IgG2b Fcγ fragment) [21]. Alternatively, coverslips were then saturated with 1.5% purified BSA (Sigma, Germany) in borate buffer for 5 min at room temperature. To preserve cell-surface cadherins, C2 cells were mechanically dissociated in trypsin-free conditions with PBS, 5 mM EDTA, 2% BSA on ice [21]. Cells were then plated on the different adhesion substrates in serum-free conditions and at very low density (5×10^2–5×10^3 cells/cm^2) for 2 h.

3. Results

3.1. MCAM-l and MCAM-s are targeted to basolateral and apical MDCK cell surfaces, respectively

GFP was inserted at the C-terminus of both avian MCAM isoforms and transfected into epithelial MDCK II cells (Fig. 1). These cells polarized spontaneously in vitro between day 5 and day 8 of culture. Confocal microscopy revealed that MCAM-l-GFP accumulated in basal and lateral membranes where it colocalized with E-cadherin, a marker for the lateral

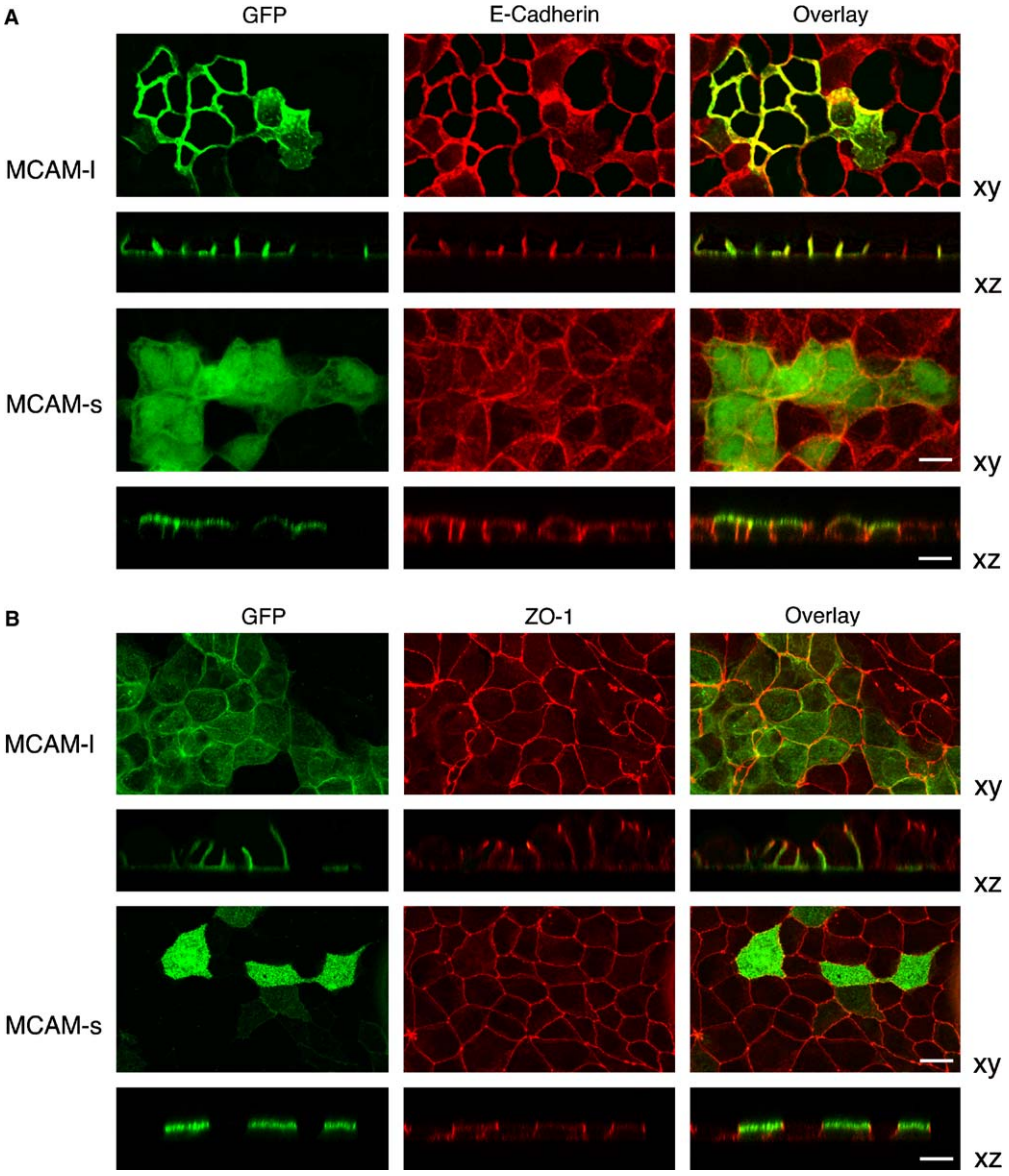

Fig. 1. MCAM-l and MCAM-s are targeted to basolateral and apical membranes of epithelial cells, respectively. MDCK cells were transfected by avian MCAM-l-GFP or MCAM-s-GFP prior to polarization. Double labeling was performed with anti-ZO-1 and anti-E-cadherin antibodies detected by TRITC or Alexa Fluor®555-conjugated secondary antibodies. Scale bar, 25 μm. (A) MCAM-l colocalized with E-cadherin on basolateral membranes but the precision of these experiments did not allow to determine if MCAM-l was targeted to adherens junctions. (B) MCAM-l as well as MCAM-s are not components of tight junctions since no colocalization were detected between MCAM isoforms and ZO-1.

membrane compartment in polarized epithelial cells but not with ZO-1, a marker of tight junctions. In contrast, MCAM-s-GFP accumulated at the apical membrane and did not colocalize either with ZO-1, the marker of tight junctions. Control experiments were performed with wild-type MCAM-l and MCAM-s isoforms, immunofluorescence detection revealed similar localization and established that GFP did not perturb MCAM isoform targeting (not shown).

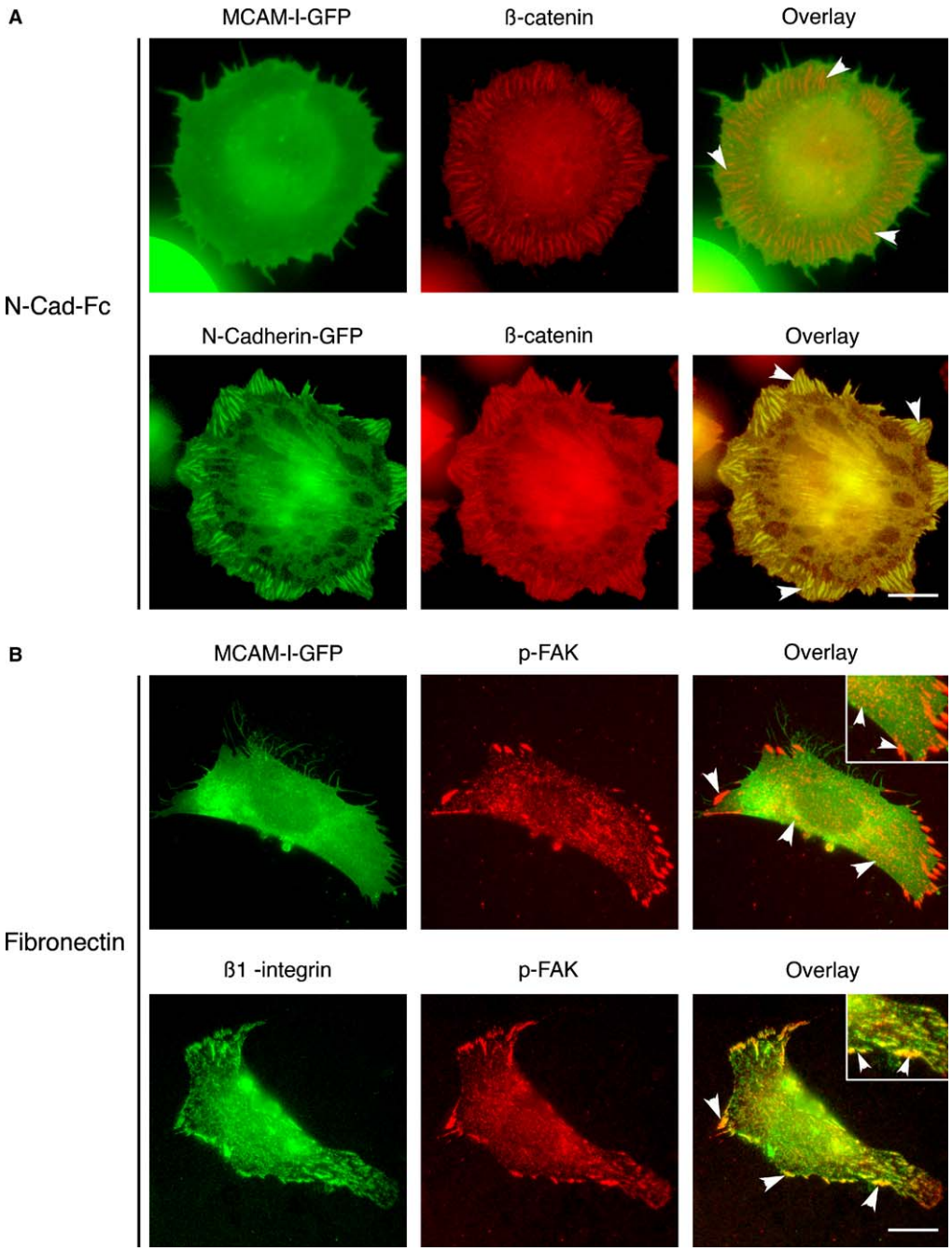

Fig. 2. MCAM-l is not a component of adherens junctions and focal adhesions. (A) MCAM-l is not localized at adherens junctions. N-cad-GFP and MCAM-l-GFP transfected C2 cells were spread on Ncad-Fc for 2 h and immunolabelled for β-catenin (red) and analyzed by confocal microscopy. β-Catenin as well as N-cadherin staining presented a radial distribution in lamellipodium (arrowheads) ; whereas MCAM-l was detected homogenously on the cell membrane. MCAM/β-catenin and N-cadherin co-localization was analysed on a 500 nm thin confocal section taken at the ventral side of cells. In contrast to N-cadherin and overlays revealed no colocalisation of MCAM-l with the radial distribution of β-catenin. Similar results were obtained with untagged wild-type MCAM-l (not shown). Scale bar, 10 μm. (B) MCAM-l is not localized at focal adhesions. C2 cells were spread on fibronectin for 2 h and immunolabelled for β1-integrin (green) and for phospho-FAK (red). MCAM-l-GFP transfected C2 cells were immunolabelled for phospho-FAK (red). The β1-integrin as well as phospho-FAK labeled focal adhesions; whereas MCAM-l was detected homogenously on the cell membrane. Scale bar, 10 μm. Inset: closer view of the focal contacts (arrowheads). Scale bar: 5 μm. MCAM/phospho-FAK co-localization was analysed on a 500 nm thin confocal section corresponding to the ventral side of cells. Overlays revealed no colocalisation of MCAM isoforms with focal adhesions. Similar results were obtained with untagged wild-type MCAM-l (not shown).

3.2. MCAM-l is not a component of adherens junctions and focal adhesions

Since MCAM-l colocalized with E-cadherin on basal membranes, MCAM-l could be a component of adherens junctions such as the cadherins. To address this question, we performed immmunolocalization analysis using a specific assay in which cadherin-mediated contacts were increased artificially by spreading cells directly onto a cadherin mimicking substrate as previously described [21]. MCAM-l-GFP transfected myogenic C2 cells expressing endogenous N-cadherin were allowed to attach at low density on an immobilized Ncad-Fc. β-Catenin and N-cadherin were recruited in radial structures linked to actin cytoskeleton and mimicking adherens junctions, also named cadherin adhesions. MCAM-l-GFP β-catenin double labeling revealed that MCAM-l homogenously distributed on the lamellipodium membrane was not further accumulated excluded in β-catenin-positive radial structures (Fig. 2). This experiment showed that MCAM-l is not recruited in actin/catenin-cadherin complexes.

In order to determine the possible involvement of MCAM-l in focal adhesions, MCAM-l-GFP C2 transfected cells were seeded on fibronectin and focal adhesions revealed using an anti-phospho-FAK antibody (Fig. 2). Double staining of MCAM-l-GFP and phospho-FAK (or vinculin, not shown) showed that MCAM-l was also excluded from focal adhesions. C2 cell transfection of wild-type MCAM-l led to similar results, indicating that the GFP tag did not influence MCAM localization with actin/catenin cadherin complex or with phospho-FAK (not shown).

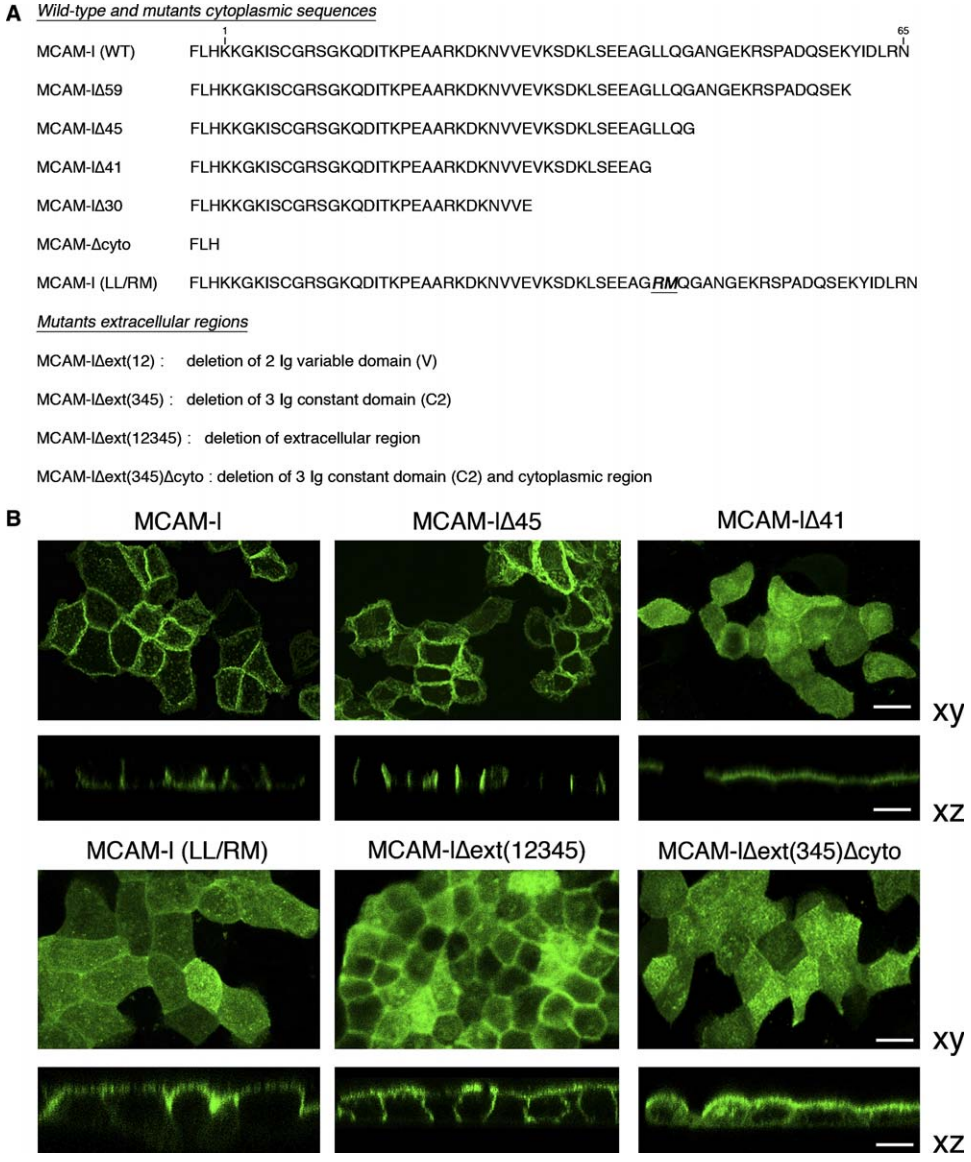

Fig. 3. The dileucine motif 41-42 of the cytoplasmic domain targeted MCAM-l to basolateral membranes of MDCK cells. (A) Panel of cytoplasmic and extracytoplasmic mutants of MCAM-l isoform. (B) MCAM-l mutant targeting in polarized MDCK cells. MDCK cells were transfected by MCAM-l-GFP constructs prior to polarization. Cells were fixed with 4% paraformaldehyde. GFP detection with confocal microscope show that MCAM-l and MCAM-lΔ45 were addressed to MDCK basolateral membranes whereas MCAM-lΔ41 and MCAM-l (LL/RM) were targeted to apical membranes. Extracellular domain mutants of MCAM-l (MCAM-lΔext12345, and not shown) were targeted to basolateral and apical membranes. Additional mutations of the cytoplasmic region including dileucine motif increased apical targeting (MCAM-lΔext345Δcyto and not shown). Scale bar, 25 μm.

3.3. The cytoplasmic dileucine motif 41-42 is required for basolateral targeting of MCAM-l

In order to identify the motif in the cytoplasmic tail of MCAM-l responsible for basolateral sorting, we created various cytoplasmic MCAM-l mutants of MCAM-l-GFP (Fig. 3). These mutants were checked in Western blot experiments using an anti-GFP antibody and their expression on the cell surface was established by flow cytometry experiments using anti-MCAM antibodies (not shown). Mutant lacking the last 22C-terminal aminoacids (Δ59, Δ45) still localized to the basolateral membrane (Fig. 3). However, when 24 or more aminoacids were deleted (Δ41, Δ30, Δ-cyto) MCAM-l-GFP was located on the apical membrane, and showed no basolateral expression. Moreover the replacement of leucines 41-42 by arginine-methionine led to apical location of the mutant MCAM-l-(LL/RM)-GFP construct. The apical targeting of this MCAM-l-GFP chimeric mutant protein thus shows that the dileucine motif at position 41-42 of the cytoplasmic tail of MCAM-l is critical for basolateral sorting of MCAM-l. Transfection of MCAM-l-GFP mutant deleted either for Ig domains 1 and 2, Ig domains 3,4, or 5, or the whole extracellular domain led to identical results. These MCAM-l mutants were targeted to basolateral and apical membranes. Thus, MCAM extracellular domain influences also membrane targeting (Fig. 3 and not shown).

4. Discussion

In this report we show that MCAM-s and MCAM-l were addressed to apical membranes and basolateral surfaces of polarized MDCK cells, respectively. Mutants of MCAM-l-GFP chimeras allowed to establish that a single dileucine motif (41-42) conserved during evolution controls MCAM-l basolateral targeting (Fig. 4). The dileucine signal in MCAM-l is similar to targeting motifs in other basolateral proteins including numerous type I and type II cadherins [22,23], furin [24], invariant chain [25], and LDL receptors [26]. In several cases, such as furin, the dileucine motif has an acidic cluster on its carboxy terminal side shown to be important for the function of dileucine signal in basolateral targeting [22]. Such acidic cluster is absent in MCAM-l as well as in the dileucine motif of B-CAM, another V–V–C2–C2–C2 Ig molecule, which is also targeted to basolateral membranes [27]. In contrast MCAM-l dileucine motif (EExxLL) belongs to the family of dileucine signals (D/ExxxLL) functioning in endocytosis or endosomal–lysosomal targeting of transmembrane protein [28]. The leucine residues of the EXXXLL motif found in HIV Nef proteins were required for binding to adaptor protein (AP-1 and AP-3) complexes of coated vesicles, inducing an expansion of the endosomal compartment [29]. It is therefore very likely that this MCAM-l motif will also function as an endocytosis motif. In addition the YXXL (59–62) motif which is not involved in MCAM-l basolateral sorting could also be involved in the endocytosis process of this molecule [28].

Apical and basolateral targeting of MCAM-l-GFP constructs deleted for the whole extracellular domains shows that the extracellular domain influence the basolateral targeting similarly to other adhesion molecules of the Ig superfamily, CEACAM-1 and PECAM-1 [30,31]. MCAM-l lateral localization would be favored by homophilic or heterophilic binding with molecules on the adjacent cells. In addition, MCAM ectodomain was found as soluble form in the culture media of hu-

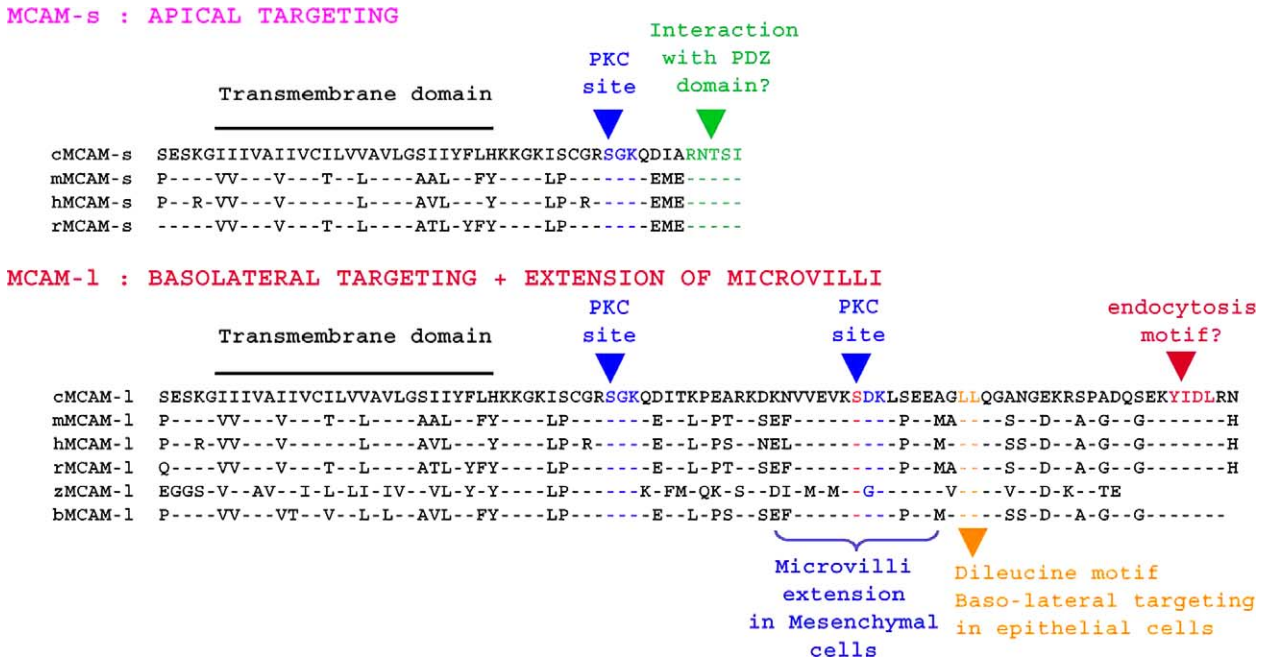

Fig. 4. Functional motifs of MCAM-l and MCAM-s cytoplasmic domains. Alignment of aminoacid sequences of MCAM-s cytoplasmic region of chicken (cMCAM-s), murine (mMCAM-s), human (hMCAM-s), rat (rMCAM-s) [2,6]. This sequence presents two conserved motifs, a PKC site (blue triangle) and its C-terminus which might interact with a PDZ domain. Alignment of aminoacid sequences of MCAM-l cytoplasmic region of chicken (cMCAM-l), murine (mMCAM-l), human (hMCAM-l), rat (rMCAM-l), zebrafish (zMCAM-l) and bovine (bMCAM-l) [2,3,6]. In addition to the PKC site encountered in the MCAM-s cytoplasmic domain, MCAM-l exhibits a second PKC site (blue triangle). Serine 32 (red) of this site is involved in MCAM-l induction of microvilli and of their extension in lymphocytes and fibroblasts (Guezguez et al., submitted). The dileucine motif (orange) is required for basolateral targeting in MDCK cells. A putative endocytosis motif YXXL is also encountered in the MCAM-l cytoplasmic tail. Note that these different motifs are conserved in vertebrates.

man endothelial cells [32,33]. Both MCAM isoforms might exhibit different susceptibility for proteolysis that could be involved in the control of MCAM membrane distribution.

Our data suggest that MCAM-l did not localize at tight and adherens junctions and confirm a previous study showing that in HUVECs MCAM did not colocalize with VE-cadherin or PECAM-1 [15]. In addition to its expression at cell–cell junction, human MCAM was also detected on the apical side of the HUVECs [15] in agreement with the expression of both MCAM-l and MCAM-s isoforms in endothelial cell lines and HUVECs [2], (Guezguez et al., submitted for publication). In addition, our adhesion assay on fibronectin suggests that MCAM-l is located outside of focal adhesions but we cannot exclude that MCAM-l is present in desmosomes as JAM-C [34]. Whatever MCAM-l precise localization on basolateral membranes, MCAM-l regulates cell–cell junctions since MCAM-l overexpression in fibroblasts decreased paracellular permeability [15] and treatment of confluent microvascular endothelial cells with an anti-MCAM antibody increased permeability to albumin in vitro [17].

In mesenchymal cells, such as fibroblasts, melanoma cells and lymphocytes, MCAM-l induces microvilli formation and extension and is expressed on these microvilli [35], (Guezguez et al., submitted for publication). We recently established that MCAM is involved in circulating cell homing. Lymphocyte MCAM-l promotes tethering and rolling by microvilli induction and rolling receptor redistribution (Guezguez et al., submitted for publication). MCAM-l basolateral targeting in epithelial cells and endothelium suggests that endothelial MCAM-l plays a role in transendothelial migration, the last step of leukocyte homing. Moreover, the different localization of MCAM-l in mesenchymal cells at top of microvilli promoting migration and at cell–cell junctions in epithelial cells participating to epithelium integrity may explain its dual roles in tumor progression. Expression of MCAM-l in mesenchymal cells such as melanoma or leukemia cells would favor invasion and metastasis and promote tumor progression [1,36]. In contrast, MCAM-l involved very likely in cohesion of mammary ductal and lobular epithelium, trophoblast and vascular endothelium, acts as a tumor suppressor in breast carcinoma as well as in infantile hemangioma [12,13,37].

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